# PHOSPHOHISTIDINE AS THE RESULT OF PHOSPHATE MIGRATION IN PHOSPHORYLATED INORGANIC PYROPHOSPHATASE FROM YEAST

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## 1. Introduction

Whereas it has been well established that many enzymic reactions involve phosphorylation of the active center, it is much more difficult to pinpoint the amino residue taking on the phosphate group. In 1962 Boyer et al. found that such an amino acid could be histidine [1]. Later, phosphohistidine was isolated from the hydrolysates of several phosphorylated enzymes. The usual procedure for this purpose is alkaline hydrolysis.

In the present paper it is shown that phosphohistidine can be isolated also from the alkaline hydrolysate of phosphorylated inorganic pyrophosphatase, but that it is the result of a migration of the phosphate group from the enzyme's active site to the imidazole ring of the amino acid.

## 2. Materials and methods

### 2.1. *Inorganic pyrophosphatase*

This was isolated from baker's yeast as described by Kunitz [2]. 3-Phosphohistidine was prepared according to Hultquist et al. [3].

### 2.2. *Phosphorylation of the pyrophosphatase*

1 ml of reaction mixture containing 6.25 nmoles of the enzyme, 1.875 nmoles of $^{32}P_i$ ($1.3 \times 10^8$ cpm) and 0.2 M acetate buffer pH 5.0 was incubated for 10 min at 3°; 0.1 ml of 1% solution of sodium dodecyl sulphate (SDS) was added and, after standing overnight, the mixture was separated on a $1.5 \times 25$ cm Sephadex G-75 column; elution was carried out at pH 7.2 with 0.1% SDS containing 0.05 M Tris-HCl buffer. Fractions of 1 ml were collected and their radioactivity estimated by a Unilux-II (Nuclear Chicago) scintillation counter. All fractions containing labelled protein were pooled, and $^{32}$P-protein was precipitated with acetone; before use, it was centrifuged and dried.

### 2.3. *Alkaline hydrolysis*

a) $^{32}$P-Protein (85,000 cpm) in 1.5 ml 3 N NaOH was hydrolyzed for 4 hr at 100° in a sealed tube. The hydrolysate was diluted with water to 10 ml, 2 mg of $Na_2HPO_4$ were added as a marker and the mixture was chromatographed on a $1.6 \times 26$ cm Dowex $1 \times 8$ ($HCO_3^-$) column, using gradient elution with 0.2–0.8 M potassium bicarbonate; total volume, 400 ml. 4 ml fractions were collected and their radioactivity measured with a Unilux-II scintillation counter. Detection of phosphorous-containing compounds was carried out in every fraction (50 µl) as described in [4].

b) The radioactive material obtained in the above section (2.3.a) (53–88 ml) was pooled, dried, hydrolyzed for 2 hr at 100° with 1 ml of 3 N NaOH and investigated as described.

### 2.4. *Digestion of $^{32}$P-protein with pronase followed by alkaline hydrolysis*

$^{32}$P-protein ($10^5$ cpm) in 0.65 ml 0.05 M Tris-HCl buffer, pH 7.2 was treated with 0.2 ml (0.2 mg) of pronase for 4 hr at 37°. Then 0.7 ml of 6 N NaOH was added and hydrolysis was carried out as described in 2.3.a; 2 mg of $Na_2HPO_4$ and 1 mg of 3-phosphohistidine were added as markers. The hydrolysate was studied as described in 2.3.a.

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The fractions giving positive ninhydrin and organic phosphate [5] tests were chromatographed in a 0.1 M $K_2CO_3$:ethanol (3.5:6.5, v/v) system with phosphohistidine and inorganic phosphate as markers. The radioactivity was determined by a Telefunken counter. The following $R_f$ values were obtained: phosphate, 0; phosphohistidine, 0.17; the radioactive compound, 0.17.

2.5. *Treatment of $^{32}$P-protein with diluted alkali followed by alkaline hydrolysis*

a) $^{32}$P-protein ($4 \times 10^4$ cpm) was dissolved in 0.6 ml of 0.5 M Tris-HCl buffer, pH 7.2; the solution was adjusted to pH 11 with 1 N NaOH and the mixture was incubated for 20 hr at 20°; then 0.3 ml of 6 N NaOH was added and hydrolysis was carried out as in sect. 2.3.a; the hydrolysate was investigated as in sect. 2.3.a and 2.4.

b) $^{32}$P-protein ($1.1 \times 10^5$ cpm) was incubated with 0.5 ml 1 N NaOH for 16 hr at 20°. Then 0.3 ml of 6 N NaOH was added and the mixture after hydrolysis was studied as described in sect. 2.3.a and 2.4.

## 3. Results and discussion

The object of this investigation was inorganic pyrophosphatase from yeast (EC 3.6.1.1). Earlier it had been established that on interacting with the substrate the enzyme is pyrophosphorylated; but that it becomes monophosphorylated in the presence of metal ions [6]. Phosphorylation of the enzyme may also be achieved by treatment with phosphoric acid [7], which is particularly convenient for studying the functional groups in the active center of this enzyme.

The enzyme may be phosphorylated at one of the following amino acid residues: serine, cysteine, dicarboxylic acids, basic acids. It had been shown earlier that SH-agents do not affect the enzymic activity of the pyrophosphatase [8] nor is it inhibited by low concentrations of diisopropylfluorophosphate [9], which indicates the absence of cysteine or of serine in the active site. Confirmation of this could be seen from the behaviour of this enzyme in solutions of varying pH. The pyrophosphatase was found to be quite labile, noticeably hydrolyzing

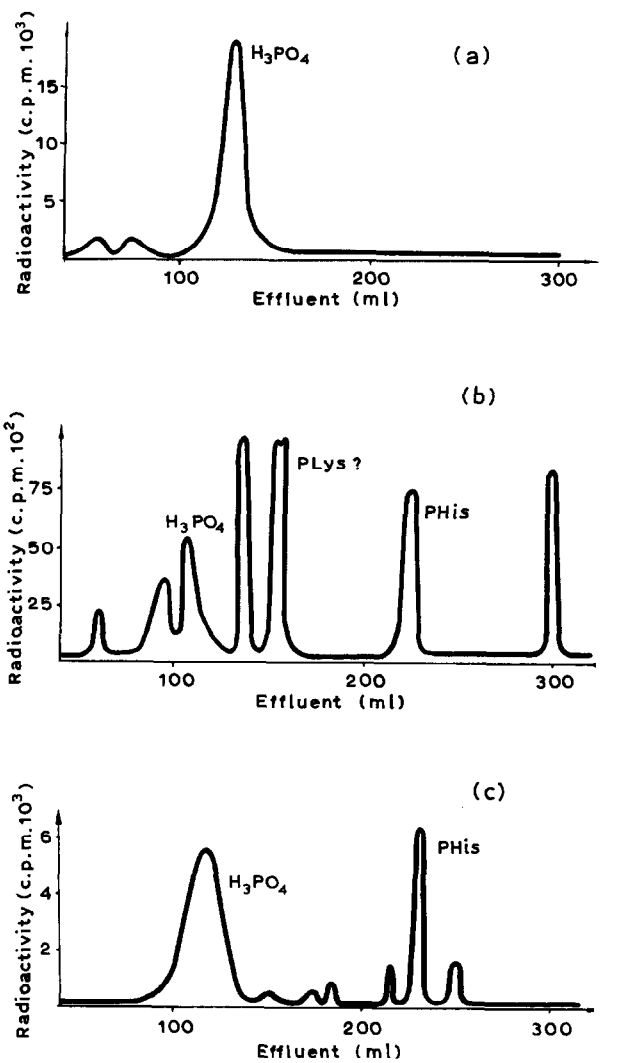


Fig. 1. Chromatography on Dowex 1 × 8 of an alkaline hydrolysate of a phosphorylated inorganic pyrophosphatase. a) After hydrolysis with 3 N NaOH (4 hr, 100°), b) after incubation with pronase (pH 7.2, 37°, 4 hr) and hydrolysis with 3 N NaOH (4 hr, 100°), and c) after incubation at pH 11, (20 hr, 20°) and hydrolysis with 3 N NaOH (4 hr, 100°).

even in neutral solutions. At pH 1 and 37° it hydrolyzes to the extent of 60% in 2 hr, and at pH 13.4 to the extent of 24% under the same conditions [7]. All of the above leads to the inference that the phosphorylated protein should contain acylphosphate or phosphoramidate bonds. In order to verify this assumption radioactive phosphorylated pyrophosphatase

was hydrolyzed with 3 N NaOH at 100° for 4 hr and the resulting products separated on Dowex 1 X 8. The elution pattern is shown in fig. 1a. One may see that the hydrolysate contains but one radioactive peak which was identified as inorganic phosphate. A small diffuse peak, which appears during the elution process somewhat prior to the orthophosphate, containing about 10% of the over-all hydrolysate radioactivity, is evidently due to incompletely split fragments. These were subjected to additional hydrolysis in 3 N alkali at 100° for 2 hr and again fractionated on Dowex 1 X 8. This yielded about 50% of the radioactivity in the form of inorganic phosphate, the other 50% consisting of unidentified products which, however, were not phosphorylated basic amino acids.

From this it follows that phosphorylated inorganic pyrophosphatase contains no phosphohistidine or phospholysine residues. However, in a number of papers, phosphohistidine which had been isolated from phosphorylated enzymes after careful treatment with alkali [10], or with proteolytic enzymes [11] and subsequent alkaline hydrolysis, had been considered to originate from the active site. It is quite possible that under these conditions the phosphate group migrates from the active site to histidine, and the isolated phosphohistidine is a secondary product of the alkaline hydrolysis.

In order to inquire into this possibility, phosphorylated pyrophosphatase was hydrolyzed after preliminary mild alkaline treatment. Pronase at pH 7.2 was used for digestion of the enzyme which was subsequently hydrolyzed with 3 N alkali at 100°. Fig. 1b shows the elution pattern from a Dowex 1 X 8 column. In contrast to alkaline hydrolysis without the preliminary treatment (fig. 1a), in this case a number of products with the phosphorus label were obtained, one of which was identified as phosphohistidine, being eluted from the column together with synthetic 3-phosphohistidine and displaying the same mobility in paper chromatography as a phosphohistidine reference.

Phosphohistidine can also be isolated from the $^{32}$P-protein if the latter is first kept at pH 11 at room temp and then subjected to alkali hydrolysis. Fig. 1c shows the chromatogram of the hydrolysate. Here also several labelled products were obtained, but the major radioactivity was detected in 2 larger peaks corresponding to inorganic phosphate and phosphohistidine. Similar results were obtained when the phosphorylated enzyme was incubated at room temp with 1 N alkali and then hydrolyzed in the usual way.

Regrettably we were unable at that moment to identify all the isolated products. One of the peaks in fig. 1b apparently corresponds to *N*-ε-phospholysine. Formation of phospholysine is quite possible under the conditions of alkaline hydrolysis and its isolation as a by-product has been described in the literature [12–14].

It follows from the above experiments that the composition of the alkaline hydrolysate largely depends upon the conditions of preliminary treatment of the phosphorylated protein. Apparently the presence of phosphohistidine in the hydrolysate is due to migration of the phosphate residue at pH 7 when histidine behaves as a nucleophile. Such rearrangement is probably responsible for the isolation of phosphohistidine from phosphorylated ATP citrate lyase as reported in [10] and of phosphoglutamic acid [15]. Our results on the alkaline hydrolysis of phosphorylated pyrophosphatase give grounds for the belief that the carboxyl group of a dicarboxylic amino acid involved in the active site of the enzyme most likely participates in the phosphorylation reaction. Support for this came from a study of the yeast inorganic pyrophosphatase from yeast with hydroxylamine and cyanide [13]. One must therefore use the utmost caution in drawing conclusions as to the source of the phosphohistidine in the alkaline hydrolysate of phosphorylated enzymes.

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